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EFFECT OF K^+ AND H^+ ON SODIUM/CITRATE COTRANSPORT IN RENAL BRUSH-BORDER VESICLES

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The uptake of citrate by renal brush-border vesicles, prepared according to the method of Vannier, occurs by Na^+-linked cotransport. It is 'positive rheogenic', i.e., stimulated by an (inside) negative, and inhibited by an (inside) positive electrical potential. The question arises whether, besides Na^+, other ions (e.g., K^+ and H^+) participate in the cotransport. As to K^+, neither an inward nor an outward directed K^+ gradient has a significant effect on the citrate movement, but at equal concentrations of K^+ inside and outside, equilibrium exchange of citrate, and to a smaller extent, the Na^+-linked net uptake of citrate, are significantly stimulated. This observation is consistent with a hypothetical model in which K^+ acts by accelerating both the empty and the fully loaded translocator. As to H^+, citrate uptake is also stimulated by decreasing extravesicular pH, an effect previously attributed to protonization of the citrate anion in the assumption that the resulting secondary citrate anion is more acceptable to the translocator site. It was found, however, that the pH effect is still apparent if the concentration of the secondary citrate is kept constant by adjusting the total citrate concentration. This is taken as an argument against the above assumption and as being consistent with H^+-linked cotransport. After the overshoot peak citrate exits slowly, and even after several hours does not attain equilibrium distribution, presumably owing to trapping by vesicular calcium.

Introduction

The uptake of citrate and other intermediates of the tricarboxylic acid cycle by the renal and intestinal brush border, similar to that of neutral substrates, has been found to occur by Na^+-linked secondary active transport, [1–5]. Surprisingly, the similarity of these cotransport processes also applies to their sensitivity to changes in membrane potential: The Na^+ cotransport of both neutral substrates and the polyvalent anions, for instance that of citrate, are 'positive rheogenic' *, i.e., stimulated by an (inside) negative electric potential difference across the plasma membrane [2,6,7]. This would imply that the translocation of citrate is tightly associated with the transfer of excess positive charge. In other words, for each mole of citrate, the sum of cationic equivalents cotransported and anionic equivalents countertransported exceeds the anion equivalents carried by citrate itself:

$$\vec{J}_+ + \overleftarrow{J}_- > J_{cit^{3-}}$$

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Abbreviations: Aces, *N*-(2-acetamido)-2-aminoethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

* Please note that we use the term 'rheogenic' in this context differently from many electrophysiologists, who use it for an electric pump functioning as a constant current source.

Since there is no evidence of countertransport with an anion, at least four cationic equivalents would have to be cotransported per citrate in order to account for its rheogenicity. The question arises whether besides Na^+, other cations, such as K^+ and H^+, are involved and with what stoichiometry. K^+ does not appear to be an essential participant, as Na^+-linked citrate transport is observed in its absence. Nonetheless, there is evidence that K^+ may partake in citrate transport, though this participation cannot simply be characterized in terms of either co- or countertransport. The marked stimulation of Na^+/citrate cotransport observed at low pH suggests that also H^+ may participate in the uptake process [8,9]. In this instance, the protons may act either by modifying the charge of the citrate anions or by functioning as a cotransported cation beside Na^+. Some evidence in favor of the latter alternative will be presented.

Materials and Methods

Isolation of brush-border membrane vesicles

Slices of cortex were prepared from essentially blood-free calf kidneys on the same day of slaughter, and stored at $-70°C$ until used. Brush-border membrane vesicles were isolated by differential precipitation using calcium ions, following the slightly modified of Vannier et al. [10,11]. The brush-border membrane fraction was enriched 11- to 12-fold in alkaline phosphatase and 14-fold for amino peptidase [12]. On the average, vesicle protein represented 2.45% of the cortical homogenate protein, as determined by the method of Lowry et al. [13]. Mitochondria, endoplasmic reticulum and basolateral membrane were not enriched in the final brush-border membrane fraction [14]. As determined by atomic absorption spectrophotometry, the vesicular material retained up to 50 nmol Ca^{2+} per mg protein.

Vesicles were preloaded with the indicated test solutions by washing the brush-border fraction twice in the appropriate solution and centrifuging at $20000 \times g$ for 60 min. Membrane were resuspended to a final concentration of approx. 5 mg protein/ml and stored in small portions at $-70°C$. Transport activity was not impaired after storage for 3 months at this temperature. All media used for membrane isolation and transport studies were cold sterilized by filtration through 0.22 μm Millipore filters and stored frozen.

Tracer uptake studies

Uptake of [^{14}C]citrate was measured at 25°C by a rapid filtration method using 0.45 μM HAWP Millipore filters [15]. Initial net uptake was measured in the presence of ion gradients and citrate gradients. Equilibrium exchange was derived from tracer uptake determinations at equal concentrations of all solutes inside and outside the vesicles. It was usually found that the citrate uptake after 20 s is almost (90%) double that after 10 s. Hence the citrate uptake within the first 10 s can be safely assumed to be linear with time and was therefore considered adequate to determine the initial rate with sufficient approcimation. Moreover, paper chromatography of vesicle extract showed no noticeable appearance of labelled metabolites after 10 s. The results were evaluated statistically using Student *t*-test for paired observations.

The composition of the solutions used in the experiments are described in the legends. The radioactivity retained by the filters was counted in liquid scintillation cocktail (National Diagnostics, Somerville, NJ) using a Tracor liquid scintillation spectrometer.

Non-specific binding and trapping by the filters and vesicles was corrected for by subtracting the value of a blank prepared for each experimental condition from the timed uptake measurements. The blank consisted of vesicle buffer and membranes added simultaneously to a 3 ml aliquot of cold 'stop' solution.

Materials

Valinomycin was purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Citrate was obtained from New England Nuclear (Boston, MA). All other chemicals used in this study were of at least analytical grade purity.

Results

General characteristics of citrate uptake by brush-border vesicles

The time course of citrate uptake into calf

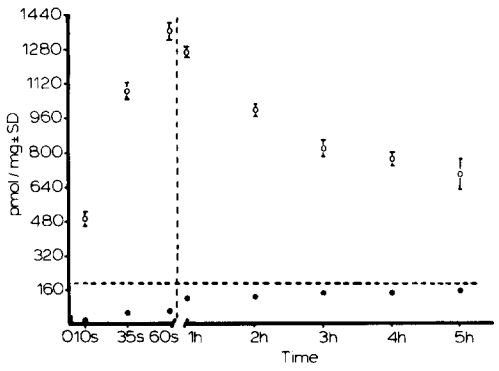


Fig. 1. The uptake of citrate vs. time in the presence of a Na^+-gradient (○), or a K^+-gradient (●). The intravesicular solution was initially 300 mM mannitol, 20 mM Hepes/Tris, pH 7.4. The extravesicular solution was either 100 mM NaCl or 100 mM KCl, 0.1 mM citrate, 100 mM mannitol, 20 mM Hepes/Tris, pH 7.4. The horizontal dotted line is to represent the uptake to be expected at equilibrium between the intra- and extravesicular space, if all intravesicular citrate were freely dissolved. The intravesicular space is derived from parallel experiments with glucose.

kidney brush-border membrane vesicles in the presence, initially, of inwardly directed Na^+ and K^+ concentration gradients is shown in Fig. 1. In the presence of the Na^+ gradient, but not the K^+ gradient, a rapid overshoot was observed. The peak was reached after about 60 s, indicating an uptake of 1300 pmol citrate/mg protein. If all this citrate were freely dissolved and if the vesicular volume per protein ratio had not changed, the intravesicular concentration at the peak would be about 0.75 mM, corresponding to a more than 7-fold accumulation. Fig. 1 shows also that after maximal uptake in the presence of Na^+, the vesicular citrate decreases very slowly and hardly falls, not even after 3 h, below about 700 pmol/mg protein. This apparent extra citrate is most likely retained inside the vesicles, as it is drastically reduced in the absence of Na^+. It is presumably trapped there by calcium, forming a soluble but non-permeant calcium-citrate complex or insoluble tertiary calcium citrate, or both as appears plausible in view of the above mentioned finding that the vesicles may retain up to 50 nmol Ca^{2+}/mg

TABLE I

EFFECT OF ANION SUBSTITUTION ON Na^+-COUPLED CITRATE TRANSPORT

The intravesicular solution was initially 300 mM mannitol, 20 mM Hepes/Tris, pH 7.4. The extravesicular solution was 100 mM Na^+, 100 mM mannitol, 20 mM Hepes/Tris (pH 7.4) and 0.1 mM citrate. The mean uptake rate of three paired determinations ± S.E. is shown as nmol/mg per min. n.s., not significant.

$NaNO_3$	3.94 ± 0.166
NaCl	3.79 ± 0.326
Na_2SO_4	2.11 ± 0.168
$NaNO_3$ vs. NaCl	n.s.
$NaNO_3$ vs. Na_2SO_4	$p < 0.001$
NaCl vs. Na_2SO_4	$p < 0.001$

TABLE II

EFFECT OF VALINOMYCIN-INDUCED, INSIDE NEGATIVE K^+ DIFFUSION POTENTIAL ON THE Na^+ GRADIENT-DEPENDENT RATE OF CITRATE UPTAKE

The intravesicular solution was initially 100 mM potassium gluconate, 100 mM mannitol, 20 mM Hepes/Tris, pH 7.4. The extravesicular solution was 50 mM sodium gluconate, 20 mM potassium gluconate, 200 mM mannitol, 20 mM Hepes/Tris (pH 7.4), 0.2 mM glucose and 0.2 mM citrate. Mannitol was isosmotically substituted for sodium gluconate in the control experiment. When present, valinomycin was 83.3 μg/mg membrane protein delivered in absolute ethanol to make up 1% v/v of the uptake incubate. For comparison, ethanol was added in the same amount in those experiments without valinomycin. The mean uptake rate for both glucose and citrate is shown as nmol/mg per min ± S.E. The number of samples analyzed is given in parenthesis after each value. n.s., not significant.

	Glucose		Citrate	
Control				
Valinomycin −	0.10 ± 0.14 (2)	n.s.	0.31 ± 0.03 (4)	n.s.
Valinomycin +	0.12 ± 0.017 (2)		0.32 ± 0.03 (3)	
Na^+-gradient				
Valinomycin −	2.43 ± 0.29 (5)	$p < 0.001$	6.4 ± 0.22 (5)	$p < 0.001$
Valinomycin +	6.81 ± 0.73 (5)		9.07 ± 0.82 (6)	

TABLE III

EFFECT OF A VALINOMYCIN-INDUCED, INSIDE NEGATIVE K^+ DIFFUSION POTENTIAL ON THE RATE OF CITRATE UPTAKE IN THE PRESENCE OF EQUAL Na^+ INSIDE AND OUTSIDE

The intravesicular solution was 50 mM sodium gluconate, 100 mM potassium gluconate, 20 mM Hepes/Tris, pH 7.4. The extravesicular solution was 50 mM sodium gluconate, 20 mM potassium gluconate, 200 mM mannitol, 20 mM Hepes/Tris (pH 7.4), 0.2 mM glucose and 0.2 mM citrate. The mean uptake rate for both glucose and citrate is shown as nmol/mg per min ± S.E., based on four independent samples for each value.

Control	Glucose		Citrate	
Valinomycin −	1.13 ± 0.19	$p < 0.001$	2.33 ± 0.11	$p < 0.001$
Valinomycin +	3.78 ± 0.51		3.1 ± 0.12	

protein. A rough calculation shows that such an amount, if all of it were available, could account for almost 25 nmol Ca to be trapped as citrate complex alone, as the dissociation constant of this complex is reportedly about 10^{-4} M [16], i.e., about the same as the presumable concentration of free vesicular citrate at equilibrium (0.1 mM). This would be about 40-times more than necessary to account for the extra retention of citrate actually found. One may infer that a major part of the vesicular calcium is not available to intravesicular citrate, being tightly bound to protein, or otherwise sequestered. In any case, the vesicular calcium would amply suffice to trap the extra amount of citrate found. We are rather confident, however, that such trapping, whatever its cause may be, will become manifest at later stages of the incubation and should therefore hardly interfere with the initial rates of citrate uptake reported in this paper, especially since after 10 s citrate is almost negligible in the absence of Na^+.

Rheogenicity of citrate transport

Na^+-linked citrate transport appears to be positive rheogenic, i.e., associated with the parallel translocation of positive charge or antiparallel translocation of negative charge. A distinction between these energetically equilent alternatives cannot be made as the charge of the unloaded translocator is not known. Rheogenicity is inferred from the sensitivity of the initial uptake rate to changes of the electric p.d. across the vesicular membrane. Changes in the electric p.d. were induced (1) by anion replacement, i.e., by using sodium salts with anions of different permeancy (Table I) or (2) by adding valinomycin to a suspension of K^+-loaded vesicles in a K^+-free NaCl medium (Tables II and III). In the anion replacement experiments, the initial uptake rate is significantly higher after substituting Cl^- by a presumably more permeant anions, NO_3^-, or SCN^- (Tables I and VI) than after replacing Cl^- by the presumably less permeant anion, SO_4^{2-}. Although the change in initial influx is less pronounced with citrate than with glucose, the uptake of both follows a similar pattern when measured in parallel with the same preparation (data not shown). The initial influx of citrate, with an NaCl gradient, is also stimulated by an opposing K^+ gradient in the presence of valinomycin, as shown in Table II. This stimulation is smaller than the simultaneously determined glucose influx, but still significant.

In rheogenic transport an electric p.d. alone,

TABLE IV

EFFECT OF A K^+ CHEMICAL GRADIENT ON THE Na^+-DEPENDENT AND Na^+-INDEPENDENT RATE OF CITRATE UPTAKE

The intravesicular solution was: (A) 150 mM tetraethylamonium nitrate (TEA)NO_3, 20 mM Hepes/Tris, pH 7.4; (B) 75 mM $TEANO_3$, 75 mM KNO_3, 20 mM Hepes/Tris, pH 7.4. The extravesicular solutions contained 20 mM Hepes/Tris (pH 7.4.), 0.1 mM citrate and the salts shown below. The rate of citrate uptake is given as nmol/mg per min ± S.E. The difference between the citrate uptake rates in the presence of Na^+ and that in the presence of either K^+ or TEA^+ is significant ($P < 0.05$) throughout. The number of samples analyzed is given in parenthesis afte each value.

	Extravesicular solution		Citrate uptake rate
A	1 (TEA)NO_3	150 mM	0.312 ± 0.028 (12)
	2 KNO_3	75 mM	0.333 ± 0.008 (11)
	$TEANO_3$	75 mM	
	3 $NaNo_3$	75 mM	5.09 ± 0.158 (4)
	$TEANO_3$	75 mM	
B	1 KNO_3	15 mM	0.271 ± 0.045 (4)
	$TEANO_3$	150 mM	
	2 KNO_3	75 mM	0.306 ± 0.043 (4)
	$TEANO_3$	75 mM	
	3 $NaNO_3$	75 mM	4.75 ± 0.155 (4)
	KNO_3	15 mM	

TABLE V

EFFECT OF K^+ ON THE EQUILIBRIUM EXCHANGE RATES OF GLUCOSE AND CITRATE DETERMINED SIMULTANEOUSLY

Intra- and extravesicular solute concentrations were equal consisting of 100 mM mannitol, 20 mM Hepes/Tris (pH 7.4), 2 mM citrate and the salts indicated below. Labeled citrate was initially present only in the extravesicular solution and its uptake at 10 s was used in the exchange rates shown in nmol/mg per min ± S.E. The number of samples analyzed is given in parenthesis after each value.

	Test solution	Glucose	Citrate
1	Choline Cl 100 mM	2.25 ± 0.03 (5)	0.96 ± 0.06 (5)
2	Choline Cl 50 mM KNO_3 50 mM	1.48 ± 0.01 (5)	0.98 ± 0.11 (5)
3	Choline Cl 50 mM $NaNO_3$ 50 mM	7.52 ± 0.28 (4)	10.13 ± 0.41 (5)
4	$NaNO_3$ 50 mM KNO_3 50 mM	6.11 ± 0.04 (4)	14.885 ± 0.6 (4)

$p < 0.05$

Glucose	Citrate
1 vs. 2, 3, 4	1 vs. 3, 4
2 vs. 3, 4	2 vs. 3, 4
3 vs. 4	3 vs. 4

without a chemical p.d. of Na^+, should suffice as a driving force. This is confirmed by the experiment presented in Table III, which demonstrates the effect of an outward K^+-gradient in the presence of valinomycin and Na^+ at equal concentration on both sides of the vesicle membrane. Again, the effect on initial citrate influx is smaller than the corresponding one for glucose influx, but still significant.

Effect of K^+ on citrate transport

(Positive) rheogenicity implies that more cations are cotransported or anions countertransported per citrate than would neutralize the translocation of the anionic charges. Since positive rheogenicity is observed even with the vesicles free of anions other than that of the buffer (Hepes), countertransport of citrate with another anion is unlikely to contribute to it. Hence it may be assumed that

TABLE VI

SENSITIVITY OF Na^+/CITRATE COTRANSPORT TO ANION SUBSTITUTION IN THE PRESENCE AND ABSENCE OF K^+

The intravesicular solution was initially 300 mM mannitol, 20 mM Hepes/Tris, pH 7.4. The extravesicular solution was 50 mM Na^+, 200 mM mannitol, 20 mM Hepes/Tris (pH 7.4) and 0.2 mM citrate. 200 mM mannitol was replaced by 100 mM potassium gluconate in the designated experiments. The mean uptake rate is shown as nmol/mg per min ± S.E., based on four independent measurements for each value. The difference between the NaSCN values in the left and right column, respectively, is significant ($p < 0.001$).

Sodium salt	Rate of citrate uptake			
	$(K^+)_i = (K^+)_0 = 0$		$(K^+)_i = (K^+)_0 = 100$	
NaSCN	7.92 ± 0.17	$p < 0.001$	11.62 ± 0.32	$p < 0.001$
NaCl	6.04 ± 0.02	$p < 0.02$	7.37 ± 0.60	$p < 0.001$
Na_2SO_4	4.36 ± 0.82		5.42 ± 0.83	

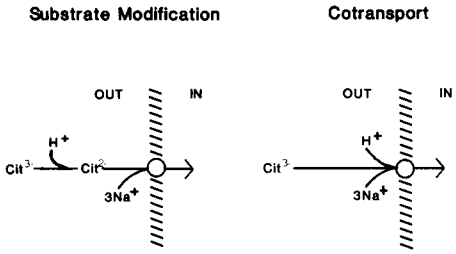


Fig. 2. H^+-effect on anion transport, which may come about by two alternative mechanisms: (a) by protonation of the anion (substrate modification); (b) by protonation of the translocator site (cotransport).

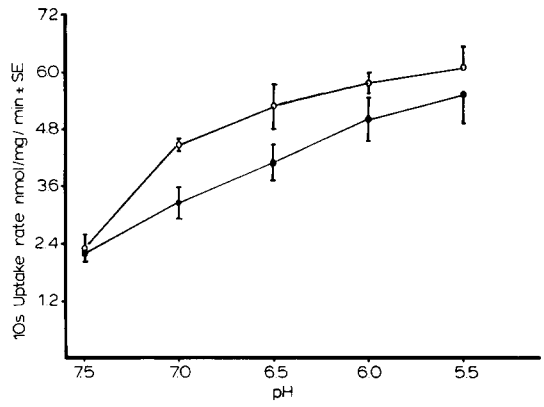


Fig. 3. Effect of citrate substrate composition on the 10 s rate of uptake at varying pH. The extravesicular solution contained 100 mM NaCl, 100 mM mannitol. Extravesicular buffers were as follows: pH 5.5, 20 mM Mes/Tris; pH 6.0, 20 mM Mes/Tris; pH 6.5, 20 mM Aces/Tris; pH 7.0, 20 mM Hepes/Tris; pH 7.5, 20 mM Hepes/Tris. The intravesicular solution was initially 300 mM mannitol, 20 mM Hepes/Tris, pH 7.4. The solid circles indicate conditions where total citrate is constant at 0.1 mM; the open circles indicate conditions where total citrate was adjusted so as to maintain divalent citrate constant at 0.1 mM for each pH value. Further information is given in Table VII.

the translocation of citrate is associated with an excess of cationic charges. The question arises whether, besides the obviously obligatory Na^+, other cations also may participate, for instance K^+ and H^+. As to K^+, it could act either vy co- or by countertransport. As seen in Table IV, however, an outward chemical gradient of K^+ (in the absence of valinomycin and in the presence of highly permanent anion to suppress any diffusion p.d.) has neither by itself, nor in combination with an inward Na^+ gradient, appreciable effect on citrate uptake, so that a true counterflow effect of K^+ appears to be absent in this system. On the other hand, an inward K^+ gradient without Na^+ has no appreciable, and in the presence of Na^+ only a very small effect, which is hardly sufficient to indicate a cotransport effect of K^+.

The presence of K^+ at equal concentrations inside and outside, however, does stimulate equilibrium exchange of citrate, in contrast to that of glucose under the same condition. This is seen in

TABLE VII

CONCENTRATIONS (IN mM) OF THE SPECIES OF CITRATE AT VARIOUS PH UNDER THE CONDITIONS THAT EITHER CONCENTRATION OF TOTAL CITRATE (I) OR THAT OF SECONDARY CITRATE (CIT^{2-}) REMAINS CONSTANT

The concentration of undissociated citrate (Cit H_3) is negligible.

pH	5.5	6.0	6.5	7.0	7.5
Concentration of total citrate constant I					
Cit^{3-}	0.100	0.028	0.056	0.080	0.093
Cit H^{2-}	0.076	0.068	0.043	0.020	0.007
Cit H_2^-	0.014	0.004	0.001	0.001	0.001
Cit (total)	0.100	0.100	0.100	0.100	0.100
Concentration of secondary citrate (Cit^{2-}) constant					
Cit^{3-}	0.013	0.041	0.129	0.407	1.288
Cit H^{2-}	0.100	0.100	0.100	0.100	0.100
Cit H_2^-	0.019	0.006	0.002	0.001	< 0.001
Cit (total)	0.132	0.147	0.231	0.508	1.388

Table V, which also shows that this effect of K^+ depends on the presence of Na^+, though not on an Na^+ gradient. Also, Na^+-linked net uptake of citrate (Table VI) is stimulated, but to a smaller extent, when K^+ is present at equal concentration on both sides of the membrane. It is seen that the presence of K^+ does not abolish the sensitivity of citrate uptake to the permeancy of the anion of the sodium salt. Hence K^+ does not appear to alter the rheogenicity of Na^+-linked citrate transport, contrary to what it should be expected to do in the case of counterflow.

Effect of H^+ on citrate transport

The Na^+-dependent uptake of citrate is markedly stimulated by lowering the pH [8,9]. This has been interpreted to indicate that not the trivalent, but the divalent, and possibly the monovalent species of citrate is the adequate substrate of the transport system. Alternative hypotheses would be that H^+ acts directly on the transport mechanism, e.g., by functioning as a coion, binding to the translocator in addition to Na^+, thereby promoting citrate transport by an affinity and/or velocity effect, or that it affects the transport barrier otherwise (Fig. 2). To differentiate between these two alternatives, the total citrate concentration was adjusted to the pH tested, so as to keep the concentration of (secondary) $citrate^{2-}$ constant, based on the extended Henderson-Hasselbach equation (Table VII). It is seen from Fig. 3 that at constant $citrate^{2-}$ the stimulation of citrate uptake by lowering pH is hardly diminished, whereas it should be expected to disappear if secondary citrate were the only transportable species, and if a change in pH had no other effect than changing the concentration of this species. One might argue that, in addition to secondary citrate, primary citrate may also be accepted by the translocator. For two reasons this is not a likely explanation: First, under the above conditions of constant secondary citrate, primary citrate is present only at a very small concentration, about one percent that of secondary citrate. Second, although this concentration does change with pH as is to be expected, such a change does not show any correlation with the citrate uptake rate: The change is smallest when that of the uptake rate is greatest and vice versa. Even when the sum of secondary and primary citrate is maintained constant, the stimulation by lowering pH is observed (data not shown). Hence, the experimental results obtained so far argue in favor of an H^+-effect via cotransport rather than merely via substrate modification.

Discussion

The experiments reported in this paper confirm the finding by Wright et al. [1] that the transport of citrate into renal brush border vesicles depends on a Na^+ gradient. The Na^+ gradient cannot be replaced by a gradient of K^+ or by another cation. It has also been confirmed that this sodium linked citrate transport is positive rheogenic, as it is stimulated by increasing the (inside) negative potential, and as excess of citrate transport depolarizes the electrical potential [6]. Since at neutral pH the citrate anion is almost exclusively trivalent, positive rheogenicity indicates that the sum of cotransported cationic charges and of countertransported anionic charges exceeds the number of anionic charges carried by the citrate. As there is no evidence of anionic countertransport, rheogenicity being observed when no anions other than buffer (Hepes) are present inside, it follows that at least four cationic charges are cotransported per citrate molecule.

The question arises whether besides Na^+ other cations are involved, i.e., K^+ and/or H^+. Countertransport with K^+ has been postulated for various Na^+-dependent systems which are stimulated by an outward downhill gradient of K^+, for instance, of neutral amino acids into Ehrlich cells [18] or sugars into brush border vesicles of the intestine [17] and, more recently, of glutamate transport into renal brush border vesicles [20–22]. This was based on the assumption that potassium preferentially binds to the unloaded translocator, thereby accelerating the reorientation of the translocator site from the inside to the outside position [23]. In positive rheogenic systems, however, true countertransport of K^+ is easily confused with the effect of K^+ diffusion p.d. Enhancement of the K^+ stimulation by valinomycin is usually considered to argue in favor of the latter and against countertransport, which was therefore dismissed for the amino acid transport systems mentioned above [24]. Also cotransport with K^+ has been reported,

such as in the furosemide-sensitive Na^+-linked Cl^- transport [25] and in phenylalanine transport [26]. Though countertransport of K^+ should counteract positive rheogenicity the opposite effect of K^+ has been reported for glutamate transport [20]. Namely that an outward K^+ gradient appeared to be required for positive rheogenicity to appear in glutamate transport.

In the present system of citrate transport, neither pure countertransport nor pure cotransport of K^+ could be verified. Neither an outward K^+ gradient in the absence of valinomycin, nor an inward K^+ gradient, with or without the presence of Na^+, appreciably stimulates citrate uptake. On the other hand, the presence of equal concentrations on both sides of vesicle membrane, in the presence of Na^+, significantly stimulates equilibrium exchange as well as the net transport of citrate in the vesicles. The observation that the latter effect is significantly greater with NO_3^- than with the less permeant SO_4^{2-}, is consistent with the previously mentioned assumption that the participation of K^+ does not diminish or abolish the positive rheogenicity of this cotransport, contrary to what should be expected if K^+ acted by countertransport only.

The observation that K^+ stimulates citrate fluxes appreciably only in the absence of a gradient may surprise, but would be consistent with the hypothesis that K^+ interacts with both the loaded and the unloaded translocator, thereby facilitating simultaneously both the forward and the backward step of the transport cycle, as illustrated by the model in Fig. 4. Accordingly, an inward K^+gradient would tend to drive the loaded translocator in the forward direction, but at the same time counteract the back reaction of the unloaded translocator. Similarly, an outward K^+ gradient would drive the back reaction of the unloaded translocator but simultaneously oppose the forward reaction of the loaded translocator. Stimulatory effect of K^+ gradient on the two translocator forms would, at least partly, defeat each other, and as a consequence the overall citrate translocation would not be affected significantly, If, however, K^+ is equally distributed between both sides, both forward and back reaction would be facilitated, and hence both net transport and equilibrium exchange of citrate accelerated, as has been found. Whereas interaction of K^+ with the loaded translocator alone would suffice to account for the acceleration of equilibrium exchange, it would not for that of net transport. A quantitative approach to this problem is given in the appendix.

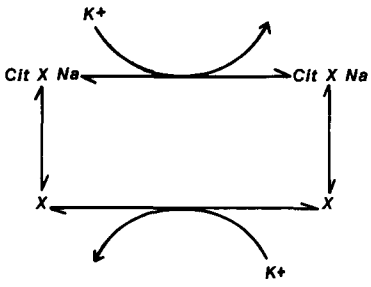


Fig. 4. Model of hypothetical K^+ effect on citrate transport. K^+ is postulated to bind to, and thereby accelerate, both the ternary complex (Cit X Na) and the empty translocator (X).

Na^+-linked citrate transport, unlike succinate transport [8], is strongly stimulated by increasing the extravesicular H^+ concentration. As with H^+-stimulated enzyme systems, the question arises whether the protons act by protonizing the substrate, thereby making it more acceptable to the translocator, or whether they act on the translocation directly, for instance via cotransport. The first alternative implies that not the trivalent citrate anion but rather the divalent citrate anion represents the translocated species [8]. Such an explanation seem plausible if citrate it transport by the succinate carrier, which is reported to be pH insensitive [8]. To test this hypothesis, we varied the pH but at the same time kept the concentration of the supposedly adequate substrate species, namely secondary citrate, constant. This can be achieved by adjusting the concentration of the total citrate [19]. If the secondary citrate were the true substrate the effect of varying pH should disappear as long as this species is kept constant. This turned out not to be the case, especially in the low pH range where the pH effects are still as strong as with total citrate being kept constant (Fig. 4). One might argue, as did the above-mentioned authors [8] that, besides secondary citrate, also primary citrate is to some extent accepted by the translocator. In the above experiments, however, the concentration of primary citrate was very low at all pH values tested, only one percent of the secondary citrate or less. Unless the affinity of the

primary citrate to the translocator is very much greater than that of the secondary citrate, the variation of the primary citrate concentration at constant secondary citrate could hardly have much effect on the pH dependence. Moreover, the correlation between the primary citrate concentration and the pH effect is very poor: the latter is the strongest in just the same pH range were the primary citrate changes the least, and vice versa. Finally, in an experiment in which the sum of both primary and secondary citrates was kept constant, also by appropriate adjustment of the total citrate concentration, the pH effect still remains, though it is somewhat weaker than that in the previously tested conditions. This may be explained by the fact that at higher pH the concentration of the tertiary, trivalent citrate inordinately increases, thus making the pH sensitivity more difficult to detect.

Two other, less likely, explantations of the pH effect may be briefly mentioned: (1) In the presence of Na^+, H^+-antiport, a pH difference may counteract the intravesicular rise in $[Na^+]$, thus maintaining the initial Na^+ gradient for a longer period of time. However, within the first 10 s the intravesicular Na^+ hardly rises enough to allow for a significant effect of the Na^+, H^+ antiporter. (2) The H^+ may discharge negative groups at the cell surface that otherwise tend to prevent citrate anions from approaching the transport sites by electrostatic forces. We believe, however, that such surface groups are too few and too weak to be of significance in this respect.

At face value, the above observations support, but do not prove, the hypothesis that H^+ acts by cotransport, and that the trivalent citrate is the adequate substrate for transport system. Accordingly, one would expect that at constant pH the uptake would rise with increasing concentration unless the latter approaches saturation values. Such a rise could, however, not always be demonstrated in the present experiment. For instance, at pH 7.5, the uptake change from 2.2 to 2.3 mol/g per min only, while the concentration of tertiary citrate varies between 0.09 and 1.3 mM, i.e., 13-fold. Also, at the other pH values tested, the correlation between uptake and concentration of Cit^{3-} is rather poor. This lack of correlation, which may be due to the fact that the values compared with each other had to be taken from different experments, does, however, not favor the alternative assumption that secondary citrate is the adequate substrate.

Appendix

K^+ effect on Na^+-linked substrate transport

The interpretation given above is intuitively plausible, but its verification requires further experiments, specifically designed for this purpose. Its plausibility, however, may be supported by showing mathematically that the observations can be predicted on the basis of the assumed model. As a complete mathematic equation, based on a general model, would be premature at the present stage of experimental evidence, equations based on a 'minimal model', in which all features are omitted that are not essential for the basic principles, will be illustrated. As an example, we use a cotransport system for the solutes A and B, such as has been amply discussed elsewhere [23], based on the following simplifying assumptions: (1) symmetry, with respect to affinities and rate coefficients; (2) quasi-equilibrium, between bound and dissolved ligands at either boundary of the barrier; (3) absence of electrical effects. In the present derivations, we also assume here that concentration and translocation of the incomplete (binary) complexes, AX and BX, can be neglected beside those of the completely loaded (ABX) and the unloaded (X) translocator, as implied by very strong 'affinity' and 'velocity' effects.

To incorporate the postulated effects of the third solute, C (which may stand for K^+), we expand the model by assuming that C binds to the ternary complex as well as to the unloaded translocator, thereby significantly accelerating the translocation of each. For the sake of simplicity, we assume that the affinity of the third solute, C, is the same for both ternary complex and unloaded translocator, respectively. These affinities are expressed by the following dissociation constants:

$$K_{AB} = \frac{a \cdot b \cdot x}{abx}$$

$$K_C = \frac{c \cdot x}{cx} = \frac{c \cdot abx}{abcx}$$

The small letters represent the concentrations of the species concerned.

Also with respect to the velocity coefficients (P), we introduce some permissible simplications, namely that they are the same for the ternary complex and the empty translocator:

$P_{AB} = P_0$, in the absence of C, and

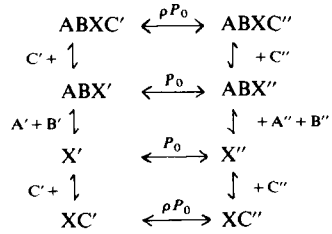
$P_{ABC} = P_C$, in the presence of C

The activating effect of C is expressed by an acceleration factor (ρ) assumed to be the same whether the translocater is loaded by A and B, or not, so

$$P_{ABC} = \rho \cdot P_{AB} = \rho \cdot P_0$$

$$P_C = \rho \cdot P_o$$

The model



P_0, rate coefficient of mobile states of translocator (without C (ABX and X).
ρP_0, rate coefficient of mobile states of translocator with C (ABXC and XC).
x_T; the sum of all possible states of each translocator, i.e.,

$$x_T = x' + x'' + ax' + ax'' + abx' + abx'' + xc' + xc'' + abxc' + abxc''$$

For reasons of simplicity we neglect ax' and ax''.

I. Initial rate of A-transport (J_A^0) (a'', $b'' = 0$)

$$J_A^0 = P_o x_T (K_C + \rho c')(K_C + \rho c'') a' \cdot b' / (K_A K_{AB} + a' \cdot b')[(K_C + c')(K_C + \rho c'') + (K_C + \rho c')(K_C + c'')]$$

This may be reduced to the following borderline conditions:

(1) C on *cis* side only ($c'' = 0$)

$${}_1J_A^0 = P_0 x_T \frac{(K_C + \rho c') a' \cdot b'}{(K_A K_{aB} + a' \cdot b')[2K_C + (1+\rho) c']}$$

(2) C on *trans* side only ($c' = 0$)

$${}_2J_A^0 = P_0 X_T \frac{(K_C + \rho c'') a' \cdot b'}{(K_A K_{AB} + a' \cdot b')[2K_C + (1+\rho) c'']}$$

(3) C on both sides ($c' = c'' = c$)

$${}_3J_A^0 = P_0 x_T \frac{(K_C + \rho c) a' \cdot b'}{2(K_A K_{AB} + a' \cdot b')(K_C + c)}$$

It is seen that ${}_3J_A^0$ is greater than ${}_1J_A^0$ or ${}_2J_A^0$, provided that $\rho > 1$.

II. Equilibrium exchange ($a' + a'' = a$, $b' = b'' = b$, $c' = c'' = c$)

(1) $c = 0$

$${}_1J_A = P_0 x_T \frac{a \cdot b}{2(K_A K_{AB} + a \cdot b)}$$

(2) $c \neq 0$

$${}_2J_A = P_0 x_T \frac{(K_C + \rho c) a \cdot b}{2(K_A K_{AB} + a \cdot b)(K_C + c)}$$

It is seen that J_A at $c \neq 0$ is greater than J_A ($c = 0$) provided that $\rho > 1$.

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